METHODS FOR DISCOVERING LIGANDS

BACKGROUND OF THE INVENTION.

~~INTRODUCTION~~

1. Field of the Invention

Interactions between molecules form the basis
of most biological processes; understanding these
interactions is important for the development of
applications in basic research and medicine. For
example, many drugs act by binding to specific receptor
molecules. The task of finding ligands that bind to a
given target with high specificity and affinity is
often difficult and though the introduction of
combinatorial chemistries will make this task easier,
it is likely that single ligands for a single
biological target may not be effective enough for some
purposes; for example, where the aim is to block
completely a specific process.

The present invention describes novel ways of
discovering combinations of ligands which act together
to produce more specific and stronger interactions than
can be achieved by a single ligand.

There are two distinct ways in which ligands
could act cooperatively:

- Many biologically important macromolecules
or macromolecular assemblages, such as proteins and
RNA, are held in their active conformation by
intramolecular interactions based on weak forces.
Binding one ligand to the molecule partially opens its
structure, and, as we will show, may expose it to other
ligands which cannot bind in the absence of the first
ligand. These additional ligands will reinforce the
attenuation of the target molecule.

2. Description of Related Art

- Many biological processes occur as a
result of a series of reactions, each one dependent on

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a different macromolecule; for example, most of the pathways that produce metabolites involve a series of steps catalysed by a number of enzymes. Each enzyme could be targeted by a different ligand to produce a greater effect on the flux through the pathway than would be produced by any one. A further benefit of using combinations of agents is that it would prevent the development of resistance to the therapy; it is a major problem in the use of antibiotics and anticancer agents that, after a time, resistance develops as a result of mutation. If the agents comprised a mixture that targeted different molecules in the cell, multiple mutations would be required to produce resistance. Clearly, the chance of a cell undergoing two random mutations that coincide two produce resistance to two agents is much less likely than the single mutation required to overcome the effects of a single agent.

SUMMARY OF THE INVENTION

~~THE INVENTION~~

The invention provides a method of designing a reagent, which method comprises providing a target, applying the target to ligands which form an array on a solid surface, observing interaction between the ligands and the target, and using the observation to design a reagent to interact with the target.

The invention also provides a method of determining combinations of ligands specific for a target, which method comprises the steps of:

a) binding at least one ligand to the target, to form a target complex,

b) applying the target complex to other ligands which form an array on a solid surface, under conditions which allow interaction between the other ligands and the target complex, and

c) identifying at least one other ligand which interacts with the target complex.

A target is a polymeric molecule having an intramolecular structure such as a nucleic acid (DNA or RNA) or a protein or carbohydrate, or a macromolecular assembly such as a membrane. Targets which are compound molecules such as glycosylated nucleic acids are envisaged. A ligand is a molecule capable of interaction with a target which interaction can form the basis of therapeutic intervention or a biological test. The nature of the interaction is not material to the invention, and may for example be hybridisation or an immune reaction or any other specific binding reaction including covalent binding. Examples of ligands are oligonucleotides, peptides, steroids and glycosides.

The invention is particularly concerned with targets that have secondary or tertiary structure. So far as nucleic acids are concerned, RNA targets often have more structure than DNA targets, and are accordingly preferred. Interaction between a target and ligands on a solid surface is preferably effected under conditions such that the secondary or tertiary structure of the target is retained. For hybridisations between polynucleotide sequences for example, such conditions are well known and are different from the more stringent conditions generally used for standard DNA hybridisation reactions.

Antisense oligonucleotides

Antisense oligonucleotides and RNAs have potential as therapeutic agents, and offer one of very few methods for rational design of therapies for a range of infectious agents including viruses, bacteria and parasites, and for the treatment of cancer. For basic biological studies, they offer a way of finding a link between a gene and its functions. The need for this becomes more important as genome analysis produces

increasing amounts of sequence data from
uncharacterised genes.   In some systems, most notably
the yeasts, genes can be ~~targetted~~ *targeted* by homologous
recombination of cloned sequences, but other systems,
5   particularly higher eukaryotes, are less amenable and
antisense methods could provide simpler and more
general means of attenuating or removing gene activity.
          The main attraction of antisense agents is
their potential to target any gene of known sequence;
10   molecules can be designed to interact specifically with
DNA and/or RNA molecules and interfere with expression
by blocking replication, transcription or translation
or by causing degradation by nucleases, or in the case
of ribozymes, by directly degrading the target RNA.
15   The feasibility of using antisense oligonucleotides as
therapeutic agents has been demonstrated in the case of
duck hepatitis virus where phosphorothioate analogues
were shown to cause complete inhibition of infection in
cultured cells and in animals [Offensperger et al.,
20   1993].   Other experimental tests have met with mixed
success [see James; 1991, for reent review], and it is
clear that if the full potential of the method is to
be realised, a number of problems must be solved.
Stability of the oligonucleotides to degradation can be
25   overcome by using analogues, but little is known about
the mechanisms by which oligonucleotides enter cells.
Several studies have shown that some positions in a
target sequence are much more susceptible than others
and that the major factor influencing the inhibitory
30   effect of an oligonucleotide is the strength of the
interaction with the target.   Our own work and that of
others has shown that the main factor influencing the
strength of interaction between RNA molecules and
oligonucleotides is intramolecular structure in the
35   target sequence.
          There is no reliable way of predicting the

interaction between an oligonucleotide and its target, and so the design of antisense reagents is largely based on informed guesswork. The methods described below provide a rational strategy for choosing antisense reagents based on novel experimental systems which can be used to measure the strength of interaction between a target sequence and all potential antisense reagents. The methods can be used to find those oligonucleotides or analogues which bind most effectively to a target sequence. The methods can be extended to find combinations of oligonucleotides which act cooperatively to open up the structure of the target, enhancing each other's binding. Antisense reagents to different RNAs, providing cooperative attenuation of the activity in a biological process involving more than one gene product, can readily be found by analysing the RNAs in the way described. The same methods can be used to find antisense targets in cells where nothing is known about specific gene expression. These targets may be single RNAs or mixtures.

The methods to be described provide an entirely new approach to antisense design. It can readily be seen that the methods described for systems based on nucleic acids could be adapted to any other system for which suitable chemical procedures already exist, or could be developed.

Arrays of ligands

To test interactions between a target molecule and large number of ligands, it is convenient to make the ligands on the surface of a solid substrate, where they can all be reacted and analysed simultaneously. Large arrays of synthetic oligonucleotides can be made on the surface of a glass plate or plastic sheet [Maskos and Southern, 1992a,

1992b; Southern, Maskos and Elder, 1992; Matson, Rampal and Coassim, 1994]. Hybridisation of labelled target sequence measures the strength of interaction with each oligonucleotide in the array. For the applications described here we use three main types of array:

1.   Oligonucleotides are created as lines on the surface of the substrate; target interactions with the oligonucleotides are analysed by applying a solution of the target in lines orthogonal to the oligonucleotides under conditions which will permit hybridisation to take place.   In this way multiple oligonucleotides can be analysed in parallel against several targets, or the same target under different hybridisation conditions [Maskos and Southern, 1992a, 1993a]

2.   Complete sets of oligonucleotides of a chosen short length are made by efficient combinatorial methods; for example, we are able to make all 4096 hexanucleotides on an array 6.5cm x 6.5cm in a six step synthesis (Fig. 1) [Southern, Maskos and Elder, 1992]. Such arrays can be used to explore interactions of oligonucleotides with the target even when no prior sequence information is available.   These arrays are used in two strategies described below: one describes the identification of oligonucleotides which bind to a known nucleic acid sequence with the object of finding those regions which are available for binding, and thus not bound up by intramolecular structure; the second strategy describes the identification of potential antisense targets in a population of mRNAs in a differentiated cell when no prior sequence information is available.

3.   "Scanning arrays" are made by stepwise synthesis in a reaction chamber which is progressively displaced along the surface of the substrate, introducing the precursor to the bases in the target

sequence at each step. A single procedure, equivalent
to synthesising one long oligonucleotide, creates a set
of oligonucleotides which represent the entire sequence
of the target molecule; each oligonucleotide in the set
5   represents a "window" on the sequence. The width of
this window can be predefined - typically we use a
window of 10-15 bases and it is an important feature of
the method for making scanning arrays that
oligonucleotides of all lengths from a single base to
10   this chosen length are made in the same process (Fig. 2).

Effects of intramolecular structure on ligand binding
Many biological macromolecules, including
proteins, RNA and DNA, are folded into relatively
15   stable structures which are important for biological
activity. The structure is dependent on the primary
sequence of the macromolecule, and also on weak
intramolecular interactions based on hydrogen bonding,
hydrophobic interactions etc. The macromolecule may
20   also be bound up with other molecules, as for example
would be a mRNA with proteins in a cell. A consequence
of molecular folding is that some residues in the
molecule are more accessible to interaction with
ligands than others; this is of great importance in the
25   design of drugs, which must bind to their target
molecule.
We illustrate the problem of finding ligands
that can bind to a folded macromolecule with reference
to tRNA^{phe}, a molecule with well characterised structure.
30   First, labelled RNA was hybridised to a general array
of the type $N_3X_2N_3$, under non-stringent conditions
which would retain the folded structure of the RNA
molecule. The pattern revealed that only a few regions
of the 76 base sequence were open to hybridisation
35   (Fig. 3 and the legend which describes the
construction of the array).

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A scanning array was then made in which the oligonucleotides comprised twelve sets, each of 76 oligonucleotides, ranging in length from mononucleotides to 12mers, representing the complement of the sequence of yeast tRNA^{phe}. Such an array can be made in one 76 step synthesis (Fig. 2a). Hybridisation of labelled tRNA to this array gives a pattern which shows how the oligonucleotides interact with all regions of the native molecule (Fig. 2a). In this example, the target presented to the general array of the type $N_3X_2N_3$ (where N is any one of the four bases and X is a mixture of all four bases) was so short (76 bases) that it was possible to make a scanning array of oligonucleotides representing the entire sequence. With a longer target, the general array would be used to identify regions of interest which would then be explored more thoroughly by means of one or more scanning arrays.

This experiment illustrates how secondary and tertiary structure play the dominant part in determining interactions. However, it is important to note that it is not possible to predict the strength of interaction, even in this case where the structure is known in great detail from X-ray diffraction studies. Some interactions of complementary oligonucleotides with the tRNA molecule agree with expectation: the strongest interactions are with the D-loop, the variable loop, and the 3'-end, all of which have unpaired bases. On the other hand, the 5'-end of the molecule and the anticodon loop, which we expected to be available for pairing, show only weak interactions. Similar studies, with parts of HIV RNA (Fig.2b) and with transcripts from exons of the CFTR gene showed that some interactions occur in regions where computer predictions indicate the presence of unpaired bases, but others do not, and most importantly for the design

of antisense reagents, some apparently open regions do
not interact with complementary oligonucleotides.

The strength of interaction, measured from
the intensity of hybridisation, is very variable, with
more than 10000-fold ratio of the strongest to the
weakest. It is a major benefit of the technique that
the strength of interaction can be seen immediately
from examination of the hybridisation pattern. It is
also possible to distinguish effects due to structure
in the target from those which are due to structure in
the oligonucleotides; this is important information
for making choices of oligonucleotides for antisense
applications. A striking feature of all analyses we
have done with natural RNAs is that only a very small
number of sites interact strongly with complementary
oligonucleotides, and we often find that the strength
of interaction changes abruptly as a result of adding
or subtracting a single base (e.g. the HIV-TAR analysis
shown in Fig.2b). These patterns of hybridisation,
which are crucially important for the design of
antisense reagents, could only be predicted from theory
if a great deal were known about the structure of the
RNA molecule, and if the algorithms used to calculate
the relative strength of interaction of different
oligonucleotides were greatly improved over those
available at present. There is no other experimental
system that could give the information provided by the
arrays.

**Identification of ligands which act cooperatively on a
folded target**

Studies of a number of mRNAs using the method
described above show that most bases are unavailable
for pairing with oligonucleotides. However, when a
first oligonucleotide does bind it is likely to break
internal structures, releasing other bases from

intramolecular pairing, and making them available for
pairing with antisense oligonucleotides which would not
bind in the absence of the "pioneer". This prediction
was borne out in experiments with tRNA.

5   Oligonucleotides which bound strongly to the D-loop and
the variable loop of the native RNA were identified
from their position on the scanning array. When an
excess of oligonucleotides of these sequences (solution
ligands) were added to the tRNA probe, hybridisation to

10   the scanning array showed dramatic increase in binding
to regions which bound weakly or not at all in the
absence of added oligonucleotide (Fig. 4). The process
can be repeated - oligonucleotides that bind in the
presence of the pioneer are included with it in the

15   hybridisation - yet other regions are opened up and
seen on the scanning array. Continuation of this
procedure can bring most or all of the target into
duplex with oligonucleotides. Clearly the combined
oligonucleotides identified by this rational stepwise

20   analysis will produce a stronger "antisense" activity
than can be achieved by any single oligonucleotide. A
further benefit of this as an approach to antisense
design is that multiple short oligonucleotides are
likely to be more specific to the target, to be taken

25   up more effectively by cells, to be effective at lower
concentrations [Morgan et al., 1993], and may be less
prone to degradation than longer ones. In several of
our analyses we have found that interactions involving
very short oligonucleotides - tetra-, tri- and even

30   dinucleotides- are stronger than any between long,
fully complementary oligonucleotides; it is unlikely
that these interactions are due to conventional Watson-
Crick pairing and thus represent unexpected
interactions which are revealed by the method proposed

35   here, and which are relevant to the design of antisense
reagents.

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Antisense reagents are not normally composed
of oligonucleotides made from natural nucleotides, but
from analogues. The important features are that:

1.   They should bind specifically to a target
5   sequence.

2.   They should be able to enter the target cell.

3.   They should be resistant to degradation in
the cell.

4.   They should induce either breakdown of the
10   target nucleic acid or block its function.

These criteria are matched better by some
modified oligonucleotides and/or analogues than by
natural oligonucleotides. The chemistry that is used
to synthesise analogues is readily adaptable to making
15   arrays, and so the strategy described here can be
adapted to any analogue. Furthermore, there are
numerous potentially desirable modifications,
for example the addition of moieties which would favour
penetration of the cell or enhance binding to the
20   target molecule whose effects on binding are not
readily predictable. The array methods would allow
these measurements to be carried out in a simple and
straightforward way, provided that a synthetic route
could be found to make the necessary structure on the
25   array; this is the case for most of the useful
modifications known at the present time.

Other ligand/target interactions

Clearly, the principles illustrated by
30   reference to antisense oligonucleotides could be used
to discover combinations of ligands of other types.
The essentials are:

- It must be possible to make an array of
one of the potential ligand types in which the
35   identities of the ligands occupying different cells of
the array are known.

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- It must be possible to detect the interaction of the target molecule with the ligands in the array.

5   It should be noted, however, that the target, the solution ligand and the ligands on the array need not necessarily be of the same molecular type. For example: arrays of oligonucleotides could be used in conjunction with intercalating agents in solution, and _vice versa_ to study effects of different intercalating
10   agents on oligonucleotide binding; different types of drugs could be used in solution and on the array to study interactions with a target protein.

Ligand libraries
15   The power of the approach would be extended if the solution ligands comprised a mixture of molecules with different structures.
           Again taking the example of oligonucleotides, we have found that different oligonucleotides open up
20   different regions of tRNA^{phe}, and that each interaction permits its own set of interactions with other oligonucleotides. This analysis was done with each solution oligonucleotide separately. If it were possible to identify the bound solution oligonucleotide
25   after the complex with the target were bound to the array, the solution ligands could comprise a mixture of all oligonucleotide sequences in a given size range. Ways to identify individual oligonucleotides or other analytes after they have been coupled to an array in
30   this way are described in an earlier patent application GB 93 15 847.5 filed 30 July 1993.
           The advantage of this double combinatorial approach could be very large; the number of interactions studied in a single analysis is equal to
35   the product of the number of ligands on the array and in solution. For octanucleotides on the array and in

solution, this would be $4^{16} = 4 \times 10^9$.

Linked ligands

Pairs or higher multiples of ligands act to increase binding to a folded molecule by inhibiting the competing reaction - reformation of the internal structure of the target molecule. They will also increase the rate of binding by opening the structure of the target. Both of these processes could be enhanced if the ligands were joined so that trimolecular or higher order interactions were reduced to bimolecular interactions. It is easy to imagine that a pair of oligonucleotides, including short ones such as di-, tri- and tetra-nucleotides, or other ligands, could be joined by a flexible linker that would allow them both to bind. The effect of linking the interacting moieties together could be further extended by enhancing the binding of one or more of the ligands to the target, for example, by covalent cross-linking, by chelation, by intercalation, or by charge or van der Waals interaction with the target molecule. This would allow other ligands more time to bind to the target. Linkers with other desirable qualities, such as greater cell permeability, could further improve the ligands' properties. A ligand could form the basis of a ribozyme, e.g. by having at least one portion for interacting with a target and another portion with catalytic activity.

EXAMPLES

Apparatus for making arrays

The device used to form the cell must make a good seal against the substrate on which synthesis takes place. We use glass for the substrate. Teflon is the only material we have found which makes a good

seal to glass and withstands the solvents and chemicals used in oligonucleotide synthesis. The surface of the cell must be finished before machining the walls that create the reaction chamber, and we have been able to

5   make a diamond shaped template that creates a good seal when pressed against a glass surface (Southern et al 1994). Alternatively, a circular template can be cut in a lathe in such a way that the surface tooling runs in the same lines as the cuts used

10 when it is pressed against the glass surface (Fig.2a). The depth of the cell is ca.0.5mm. Inlet and outlet ports were made by drilling 1.0mm diameter holes at the top and bottom of the circle and fitting sawn-off 19 gauge (1.1mm o.d.) syringe needles through from the

15 back. The Teflon cell was mounted on a rail fitted with a lead screw that was used to displace the glass plate relative to the Teflon reaction cell. A "G" clamp, fitted with a dished polyethylene cushion, was fixed to the rail to apply pressure to the glass plate

20 and form the seal to the reaction cell. The rail was mounted on the front of the frame of an ABI 381A oligonucleotide synthesiser so that the delivery lines normally connected to the column could be connected to the reaction cell.

25

Making arrays

        Glass plates (50 x 220 x 3mm) were first coated with a covalently attached linker [Maskos and Southern, 1993]. Plates were immersed in a mixture of

30 glycidoxypropyl trimethoxysilane/diisopropyethylamine/ xylene (17.8:1:69, by volume), heated to 80°C and held at this temperature for 9h, and then washed in ethanol and ether. In a second step, the plates were heated in neat hexaethylene glycol, containing a catalytic amount

35 of sulphuric acid, at 80°C for 10h, washed with ethanol and ether, air dried and stored at -20°C.

Oligonucleotide synthesis used standard reagents for phosphoramidite chemistry, omitting the capping step. The ABI 381A was programmed to couple bases in the order corresponding to the complement of the target sequence, with an interrupt after deprotection. The scale was for 0.2 μmol. synthesis, adjusted slightly to provide volumes that would just fill the reaction chamber.

Final deprotection in 30% ammonia was carried out in a specially constructed bomb, comprising a chamber (230 x 230 x 20mm) cut into a Nylon block (300 x 300 x 30mm), sealed by a sheet of silicone rubber (3mm thick), compressed against the rim of the chamber by clamping the whole assembly between two mild steel plates (6mm thick) using four bolts along each side of the square. After 5-8h at 55°C the bomb was cooled to 4°C before opening. The plate was then washed in ethanol followed by Tris/EDTA (0.01M, pH 7.8, 0.1% SDS) and ethanol and then dried in an air stream.

Hybridisation reactions

We have used a variety of target molecules in experiments with scanning arrays: synthetic oligonucleotides labelled using polynucleotide kinase with gamma-^{32}P, gamma-^{33}P or gamma-^{35}S-ATP to tag the 5' end; RNAs labelled at the 3' end using RNA ligase with 5'-^{32}P cytosine-3',5'-diphosphate; or transcripts of DNA fragments made from PCR amplified fragments using T7 or SP6 polymerase to incorporate α-^{32}P or α-^{35}S UTP. All of these make good hybridisation targets. Most hybridisation reactions were carried out at 4-25°C, in solutions containing 3-4.5M TMACl or 1.0 M NaCl. After hybridisation, the plate was rinsed in the hybridisation solvent and exposed through Clingfilm to a storage phosphor screen (Fuji STIII) which was then scanned in a Molecular Dynamics 400A

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PhosphorImager.

In the specific examples shown in Figures 2
and 4, experimental conditions were as follows:

tRNA^{phe} (10 pmol, Sigma) was dissolved in

5   HEPES buffer (50mM, pH7.4; 20mM MgCl$_2$, 3.3mM DTT,
1μM ATP, 10 μg/ml BSA, 10% DMSO) with cytosine-3',5'-
diphosphate (3000Ci/mmol, Amersham), and T4 RNA ligase
(9 units, Pharmacia), after 30min incubation at 37°,
the reaction was separated in a spun column and the

10   labelled RNA dissolved in 3.5M TMA (10ml).
Hybridisation was carried out by applying ca. 1ml of
the solution to the surface of the array and overlaying
a second glass plate of the same dimensions. The
"sandwich" was placed in a sealed box at 4°C for 18-

15   24h. The plates were separated, the array rinsed in the
hybridisation solvent at 4°C, and analysed as described
above.

For the cooperative experiments shown in
Figure 4, cold oligonucleotides corresponding to the D-

20   loop GCTCTCCCAACT, the variable loop GACCTCCAGATT, or
the TpsiC loop AACACAGGACCT, were incubated with the
tRNA in the hybridisation conditions for at least 18h
before being applied to the plate.

25   FIGURES

Figure 1.

An illustrative array comprising all
tetranucleotide sequences. This is one sixteenth the
size of the smallest usable array comprising all

30   hexanucleotides, which would be too complex to display
in this format. The letters along the top and down the
left hand side show the order in which base precursors
were applied in columns and rows during the synthesis
of the oligonucleotides. The letters in each cell show

35   the sequence of the oligonucleotide synthesised in that
cell. Larger arrays are made by simply ovelaying

increasingly wide rows and columns such that each
successive row or column embraces four from the
previous layer. In all cases, the array comprises all 4^s
possible oligonucleotide sequences of length s. The
5   results of hybridisation to such an array are shown in
Fig. 3. More complex arrays can be made by applying
mixtures of all four bases at certain stages in the
synthesis; for example, all octanucleotides can be made
in 4096 cells by applying four mixed bases in the two
10 central positions, to produce an array of the type
$N_3X_2N_3$. In this case, each cell comprises a mixture of
16 different octanucleotides. The results of
hybridisation to such an array are shown in Fig. 3.

15 **Figure 2a.**

        Scanning arrays are made by applying
oligonucleotide precursors to the glass substrate in a
circular patch. The first base, corresponding to the
complement of the 5' end base of the target sequence, is
20 applied to the left end of the plate, which is then
moved by a predetermined off-set, and the second base
added. The process is repeated for the whole of the
sequence. The length of oligonucleotides made on the
centre line is equal to the diameter of the cell
25 divided by the offset, in this case, diam. = 30mm and
offset = 2.5mm, giving a maximum overlap of
30/2.5 = 12. Oligonucleotides ranging in size down to
a single base are made in the segments flanking the
centre line.
30   The upper panel shows a scanning array
representing the 76 bases of tRNA^{phe}, hybridised to end
labelled tRNA. Note the strong hybridisation in the
region of the D-loop, more moderate hybridisation to
the variable loop, and very weak hybridisation to the
35 anticodon loop and the 5'-end of the molecule.

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Figure 2b.
The lower panel shows a scanning array representing 76 bases of HIV TAR element hybridised to an *in vitro* labelled transcript of the same region.
5 Note the strong hybridisation to a single 12-mer, which is tenfold stronger than hybridisation elsewhere. This 12-mer corresponds to the region of the loop which has been shown by others [Ecker *et al*., 1992] to form a pseudo-half-knot with an adjacent 12-mer.

10

Figure 3.
Hybridisation of a tRNA^{phe} to an array of the type N_3XXN_3, where N is any one of the four bases and X is a mixture of all four, so that 65536 different
15 octanucleotides are all present on the array, in cells containing mixtures of 16 related sequences. This result shows which regions of the sequence are most open to hybridisation. These can then be examined in detail on a scanning array, as shown in Figure 2a.

20

Figure 4.
Effects of including non-radioactive oligonucleotides (positions in the structure and the exact 12-mer indicated in the panel) with the tRNA
25 target in the hybridisation solution. Note the large increase in binding to regions of the sequence that do not hybridise in the absence of the added oligonucleotides, showing that these regions have been opened up. The top panel is the control with no added
30 oligonucleotide. The third and fourth panels show that adding a second oligonucleotide exposes regions that are not opened by the first oligonucleotide alone.

Figure 5.
Cooperative antisense oligonucleotides
35 against rabbit ß-globin mRNA.

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Much is known about the rabbit globin system.
It was one of the first mRNAs to be isolated and
characterised by translation <u>in vitro</u>. We have studied
it because we wish to use it to study it as a model for
5   antisense intervention. In preparation, we made a
scanning array of oligonucleotides complementary to 122
bases around the initiation codon of the mRNA (atg
marked in bold letters at bases 54-56). The folded
structure shown in the figure is that with minimum free
10   energy calculated by the energy minimisation program
mfold, and displayed by squiggles. Hybridisation of
labelled RNA showed interaction at the region
complementary to bases 46-62 marked by a thick line on
the molecule. We then made a deoxyribooligonucleotide
15   corresponding to this 17-mer. Rehybridisation of the
complex showed, as expected, no signal at the position
of bases 46-62, but new signal at bases 32-45, marked
with a dashed line. This is a surprising result, as
there is no indication from the computer prediction
20   that bases 46-62 interact with bases 32-45. On the
contrary, there is a single stranded region of nine
bases between them, which should be enough to decouple
any interaction. Thus, the experiment suggests an
interaction relevant to the design of antisense
25   reagents which could not have been predicted by energy
calculations or by examination of the computer
generated structure.

Figure 6.

30   Four arrays were made of complements to the
same sequence, a region of the human CFTR gene
(mutations in this gene are responsible for cystic
fibrosis). The arrays were made as described in
Southern <u>et al</u> (1994) on a glass substrate derivatised
35   with a hexaethylene glycol linker.
a)   natural deoxyribonucleotides tethered to the

glass through the 3' ends.

b)   natural deoxyribonucleotides tethered to the glass through the 5' ends.

c)   deoxyribophosphothioates (Note that this
5   array was exposed in the opposite orientation to the other three).

d)   ribonucleotides.

The arrays were all hybridised under identical conditions (3.5 M tetramethylammonium
10   chloride, 4°C) with the sequence CCTGGCACCATTAAAGAAAATATCATCTTTGGTGTTTCCTAT, part of exon 10 of the CFTR gene covered by the array.

The deoxyribonucleotides give essentially the same result in both chemical orientations, but the
15   analogues, the deoxyribophosphothioates and ribonucleotides, give quite different results. Although a difference may be expected, this experiments shows the difficulty in extrapolating data from one analogue to others, and further demonstrates the power
20   of the array technique in identifying candidates for antisense reagents, including analogues.

Figures 7(a)-7(c).
~~Figure 7.~~

The Rev response element (RRE) of HIV is
25   considered to be a good potential target for therapeutic intervention by antisense oligonucleotides because of its central place in the regulation of gene expression from the viral genome. However, molecular modelling in the computer, as shown (Fig. 7a), suggests
30   that this region of HIV RNA is likely to have a complex folded structure, and this has been confirmed by analysing the susceptibility of the RNA to nucleases. This structure makes it difficult to select regions for antisense targetting. We have analysed the
35   hybridisation behaviour in a two stage process. First, the labelled RNA was hybridised to "universal" arrays,

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that is arrays comprising all oligonucleotides of a
chosen length. In this experiment, we used two types
of universal arrays, one comprising all 4096
hexanucleotides (Fig. 7b), the other all 65,536

5   octanucleotides (Fig. 7c). In the latter array there
were only 4096 cells, each containing a mixture of
sixteen different octanucleotides of the general form
NNNXXNNN, where N is a defined base, and X is a mixture
of all four. These universal arrays do not indicate

10   the optimum oligonucleotide to use, as they only have
sequences of limited length, but they do reveal the
regions in the target sequence which are most available
for hybrid formation. These areas were then analysed
in more detail using scanning arrays as described

15   below. The method used to make the universal arrays
produces oligonucleotides half the length of the major
oligonucleotides in lines which intersperse them. A
surprising result of the analysis is that some of these
tri- and tetranucleotides interact strongly with the

20   target (see the lines of uniform intensity in Figs. 7a
and b). With other targets we see the same feature,
but with different oligonucleotide sequences. In one
case (Southern et al, 1994), we have seen interaction
between a target and a dinucleotide. It is likely that

25   these interactions are the result of the folding of the
target RNA, which may present short stretches of
sequence in a structure which is particularly
favourable for interaction with oligonucleotides; for
example, a stacked half helix. These observations

30   suggest a novel approach to antisense design, based on
the use of array analysis. Namely, that short
oligonucleotides, which are seen to interact in this
structure specific manner can be incorporated in the
cocktail of antisense agents specific to the target,

35   either as a component of a mixture, or as a component
of a linked composite molecule.

Figures 8(a) - 8(c).

~~Figure 8.~~

The region of the RRE analysed in Fig. 7 was also analysed on a scanning array made of oligodeoxyribooligonucleotides with 17mers as the

5   longest. Only one oligonucleotide in the Rev binding region showed strong interaction at 37°C in 3.5 M TMACl (Fig. 8a). When this oligonucleotide was bound to the target in solution and the complex again applied to the array, several sites showed interaction with

10   oligonucleotides which had not bound in the absence of the solution oligonucleotide (Fig. 8b). /The same region bound when the RRE target was analysed on an array of phosphothioate analogue oligonucleotides at 20°C in 1 M NaCl (Fig. 8c)/.

15   Figures 9(a) - 9(b).

~~Figure 9.~~

The analysis described in Fig. 8 was repeated in the presence of neomycin, a drug which is known to act by binding to RNA molecules. The presence of

20   neomycin substantially altered the pattern of interaction of RRE with some oligonucleotides. (Figs. 9a and b). In particular, regions which bind poorly in its absence bind more strongly in its presence.

Figures 10(a) - 10(b).

~~Figure 10.~~

25   Magnesium is known to have a significant effect on the folding behaviour of RNAs and we have examined its effect on the binding behaviour of tRNA using the scanning array used in the experiments

30   described in Figs. 2a and 4. We find that several oligonucleotides which do not bind in the absence of magnesium ions (Fig. 10a) do so in its presence (Fig. 10b).

Figures 11(a) - 11(c).

~~Figure 11.~~

35   The TAR element is another important target

for HIV. We have analysed its hybridisation behaviour
with arrays of natural oligodeoxyribonucleotides, and
with an array made with analogues that are commonly
used as antisense agents, 2'-O-methylribonucleotides

5   (Fig. 11c). The same region is found to hybridise in
both cases, the loop of the stem-loop, but only one or
two sequences in this region form duplex, and the exact
position of the sequence with strongest interaction is
different with each analogue. In an experiment with

10   the natural oligodeoxyribonucleotides, we selected an
antisense sequence as the "pioneer" (Fig. 2b and 11a)
and used it to open the structure. Note the loss of
binding in the region corresponding to the solution
ligand, and the additional binding at other sites.

15   When the complex was bound to the array, several
oligonucleotides which had not bound in its absence,
now gave a significant yield of duplex (Fig. 11b).

Conclusion

20   The above examples illustrate several new
approaches to the design, characterisation and
discovery of ligands made possible by analysis on
arrays on solid supports. We have shown how this
approach can:

25   - Identify regions of the target that are
open to interaction with ligands;

             - Identify combinations of ligands that act
together to give a different, usually stronger,
interaction than either alone;

30   - Discover regions of the target that are
structured in such a way as to allow them to bind in
unexpected ways, e.g. target RNAs to very short
oligonucleotides;

             The arrays and the cooperative ligands used

35   in the examples were made of a variety of chemical
types, illustrating the generality of the approach.

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